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Effective Construction of DNA Vaccines Against Variable Influenza Genes by Homologous Recombination

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We demonstrate the potential of cloning by homologous recombination as a rapid method to construct DNA molecules encoding newly developing hemagglutinins (HA) of influenza A virus. The variable parts of the HA genes were cloned into a basic construct containing the HA gene from an H3N2 strain. The recombinant DNAs thus created encode different variable domains with neutralising epitopes from four recently circulating influenza A H3 strains. The technology allows rapid production of DNA constructs for vaccines that can induce antibody and, particularly, cellular immune responses. These new constructs were also capable of conferring protection to challenge in mice. The technology may hence be a valuable tool for rapid adaptation of influenza vaccines to changes in the circulating influenza strains.

INTRODUCTION

Influenza is one of the main causes of death among the elderly in many countries (Dauer and Serfling, 1961; Eickhoff *et al.,* 1961; Stanley and Jackson, 1966; Barker *et al.,* 1982). The most cost-effective medical intervention against influenza is vaccination. Because the antigenic determinants of the viral envelope proteins hemagglutinin (HA) and neuraminidase (NA) undergo continuous immuno-selection during viral spread in the population (antigenic drift) and existing vaccines elicit only short-time protective immune responses, vaccination must be repeated annually (Fitch *et al.,* 1997). The mutation rate of the RNA encoding these proteins is 1.5×10^{-5} per nucleotide per infectious cycle, compared to 10^{-11} per incorporated nucleotide in the mammalian genome at cell division (Parvin *et al.,* 1986). Alteration of a few amino acids in the HA and NA proteins is often sufficient for immune escape. For these reasons, the market life of a vaccine is rarely more than one season, and the selection of a new vaccine involves prediction of the coming season's mutations (Parvin *et al.,* 1986).

DNA vaccines offer a novel approach to immunisation. DNA encoding a specific antigen is transferred to a host cell, in which the antigen is expressed and presented to the host's immune system, hence eliciting an immune response. The rapid progress in the field started after Wolff *et al.* reported direct gene transfer *in vivo* following intramuscular (im) injection of naked plasmid DNA in mice (Wolff *et al.,* 1990). In 1993 Robinson *et al.* reported humoral responses and protection against viral challenge following intramuscular inoculation of the hemagglutinin gene (Robinson *et al.,* 1993), and Ulmer *et al.* observed the induction of cytotoxic T cells and protective immune responses to influenza A after intramuscular injection of plasmid DNA encoding the nucleoprotein (Ulmer *et al.,* 1993).

In the present study, we examined the possibility of using cloning by homologous recombination to produce recombinant DNA vaccine vectors and the cellular expression of recombinant genes hence constructed. The variable region of the HA_1 domain from four predominant viruses of the recent influenza seasons in Sweden were successfully introduced in the backbone of a genetically different influenza A H3N2 HA gene. Also, the immunostimulatory and protective capacity of these plasmid constructs were tested in mice in this study. The hypothesis was that our technology would facilitate cloning of the antigen determinant region of an emerging influenza HA, permit correct expression, and elicit immune responses to viral challenge.

RESULTS

Cloning strategy and amino acid substitution analysis

A basic construct encoding the HA gene from the influenza A/Stockholm/6/96(H3N2) virus was prepared with standard cloning techniques. The rationale for using this strain was its predominance in Sweden during the previous influenza season. It was also antigenically identical to the influenza A H3 vaccine strain during 1997–98. When a new virus emerged [the influenza A/Stockholm/7/97(H3N2)], we

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investigated whether our DNA construct could be easily modified to represent the antigenic drift. When the cloning procedure was established, we also investigated the possibility to clone in the antigen determinant regions from the preceding season's viruses, the influenza A/Netherlands/18/94(H3N2) and A/Umeå/1/95(H3N2) into the vector handle. When a new, drifted strain appeared, the influenza A/Stockholm/5/99(H3N2) (Swedish Institute for Infectious Disease Control, Registered Annuals), the antigen determinant region from this very recent strain was also cloned into the vector handle. The variable region of the basic hemagglutinin gene construct was deleted, leaving a linear vector handle. The variable regions of the HA_1 domains from the A/Netherlands/18/94(H3N2), A/Umeå/1/95(H3N2), A/Stockholm/7/97(H3N2), and the A/Stockholm/5/99(H3N2) isolates were cloned into the A/Stockholm/6/96(H3N2) basic construct by homologous recombination, replacing the previous variable region (Fig. 1). Homologous recombination takes place in the bacterial cell (Bubeck *et al.,* 1993) when overlapping sequences in the PCR fragment (in our study, ~300 bp) recombine with homologous sequences of the vector handle. The recombination is catalysed by the host's recombination system. Recombination was confirmed by sequencing, and the approximate sites of homologous recombination were analysed by comparing amino acids in the basic construct and the recombinants (Fig. 2).

As an example, one recombinant clone was analysed in detail. After homologous recombination with the A/Stockholm/7/97(H3N2) into the A/Stockholm/6/96(H3N2) vector handle, nine amino acid substitutions were identified in the variable region that derived from the A/Stockholm/7/97(H3N2) primary isolate (Fig. 2). The amino acid substitutions caused a change in net charge from −1 to +4 in these positions. Also the aspartic acid at position 133 in the basic construct was replaced by an asparagine, followed by a glycine and a threonine. This would add a site for N-linked glycosylation at position 133 (Alberts *et al.,* 1994). Comparisons of the HA_1 from the A/Stockholm/6/96 and the A/Stockholm/7/97 strains showed that seven of the nine substitutions mapped within or immediately adjacent to the antigen determinant regions A–C and E (described by Wiley *et al.,* 1981).

Expression of recombinant hemagglutinin

Expression of a hemagglutinin was tested by immunofluorescence and its biological activity was tested by hemadsorbtion in 293 cells transiently expressing the recombinant influenza HA [A/Stockholm/7/97(H3N2)] in the pUACMVSMI2 DNA vaccination vector. Figure 3a shows the immunostaining of transfected cells at 40 h posttransfection using the H3-specific monoclonal antibody MC3A10-18 as compared to untransfected control cells. In this particular experiment, ~30% of the cells stained positive for HA. Similarly transfected cells were incubated with a standard 0.4% suspension of guinea-pig erythrocytes in PBS used for clinical diagnostics of influenza. In this experiment, ~5% of transfected cells showed adsorption of erythrocytes, whereas the remaining untransfected cells did not adsorb any erythrocytes (Fig. 3b). Agglutination of red blood cells (hemadsorption) by influenza infected cells depends on the binding of HA expressed on the cell surface to its cellular receptor on erythrocytes; sialic acid linked to protein- or lipid-bound galactose by α2,6 or α2,3 linkages (Weis *et al.,* 1988).

Immunisation and challenge

Mice were vaccinated with killed virions, the HIV rev encoding plasmid, the original HA gene [A/Stockholm/6/96(H3N2)], or HA DNA produced through homologous recombination [A/Stockholm/7/97(H3N2)]. The prechallenge antibody titres were analysed by ELISA after one booster immunisation. Mean group titres ranged between 110 and 3750 in DNA-immunised mice as compared to 650 in sera from mice immunised with killed virions (data not shown). Next the mice were challenged with the mouse adapted A/PR/8/34(H1N1) influenza strain. Mice immunised with DNA showed an increased survival rate and less body weight loss compared to mice immunised with killed virions or rev-encoding plasmids or unvaccinated animals (Fig. 4).

In a similar immunisation study, we evaluated whether a cellular immune response developed after one single immunisation with either a recombinant DNA vaccine [A/Stockholm/5/99(H3N2)], killed virions or control DNA (encoding the HIV-1 rev gene). Lymphocytes from all mice were strongly stimulated by ConA, demonstrating the viability of the cells. Four of four mice immunised with the inactivated influenza virus had a strong and specific T-cell response against influenza A/Stockholm/5/99(H3N2) in the lymphocyte proliferation assay. Four of five mice immunised with the recombinant HA gene had a specific T-cell response against the influenza A/Stockholm/5/99(H3N2) virus, showing a very high stimulation index even though there was a nonresponder in this group. None of the mice immunised with control DNA showed any indication of a specific T-cell response against influenza A/Stockholm/5/99(H3N2) (Fig. 5). Only the mice immunised with the killed virions had HA-specific antibodies after one immunisation as determined by ELISA (data not shown).

DISCUSSION

We have demonstrated that rapid cloning by homologous recombination offers a means to rapidly construct DNA plasmid vectors encoding new strain-specific hemagglutinins and that the functional protein is efficiently expressed in mammalian cells. This is potentially useful in constructing DNAs of emerging influenza strains. The vector handle approach has advantages over traditional

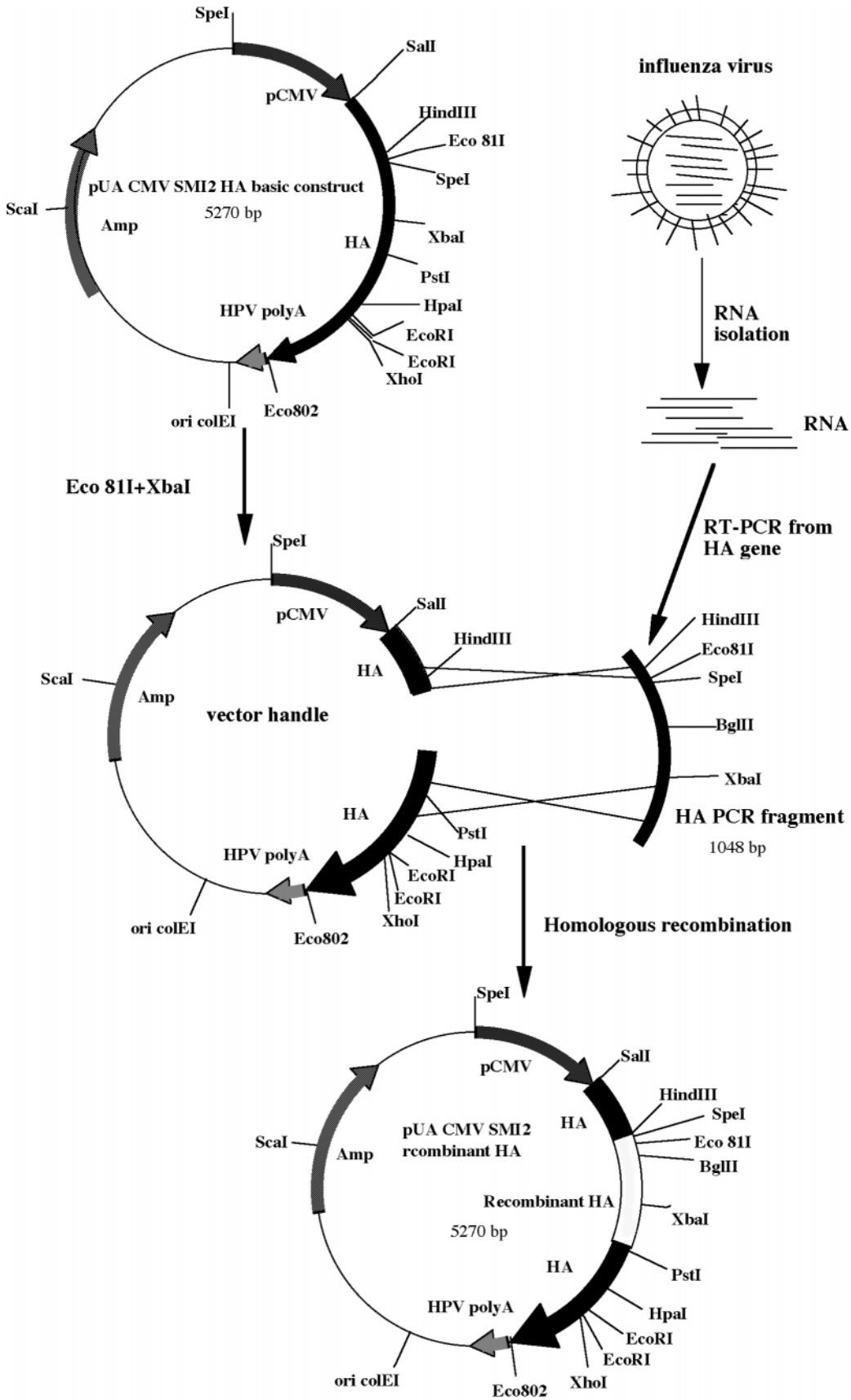

FIG. 1. Cloning strategy for rapid cloning through homologous recombination. A recombinant HA gene was constructed by homologous recombination. The variable region of the HA gene from a primary isolate was cloned into a vector handle.

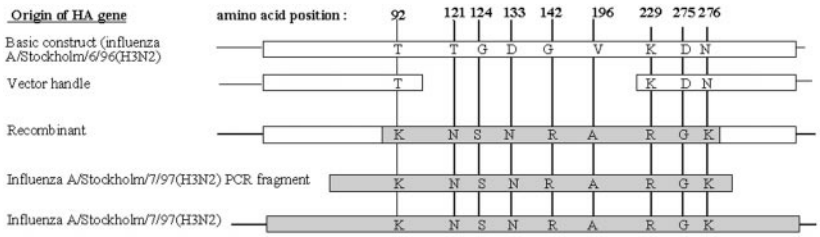

FIG. 2. Comparison of the hemagglutinin amino acid sequences encoded by the influenza A/Stockholm/6/96(H3N2) strain, the vector handle, recombinant hemagglutinin, influenza A/Stockholm/7(H3N2) PCR fragment, and the A/Stockholm/7/97(H3N2) strain. The recombinant has the neutralising epitopes from the emerging virus, the influenza A/Stockholm/7/97(H3N2) strain, in the basic construct backbone. The amino acid substitutions generated by the antigenic drift caused a change in net charge from −1 to +4 and added a site for N-linked glycosylation in these positions.

cloning, in that no compatible restriction sites are required in vector or insert and that ligation is not necessary. Additionally, in frame cloning will always be achieved. This technology could also be used to rapidly clone variable regions of RNA encoded antigens from different viral strains to form multivalent vaccines consisting of a mixture of recombinant DNA plasmids. In our study of the immune responses induced upon vaccination with these plasmids, only antibody production and specific T-cell proliferation were studied. Cytotoxic T cell assays were not included because there are no clearly identified CTL epitopes for mice in the H3 HA amino acid sequence.

Primary immunisations with killed influenza virions in humans typically give a strain-specific immunity. It was previously described that DNA immunisation appears to give a broad cross-strain protection to heterologous influenza strains after immunisation with the nucleoprotein (NP) gene (Fu *et al.,* 1993, 1997; Ulmer *et al.,* 1998) or with the NP gene together with the HA gene (Robinson *et al.,* 1999). In a ferret model system, protection against drifted

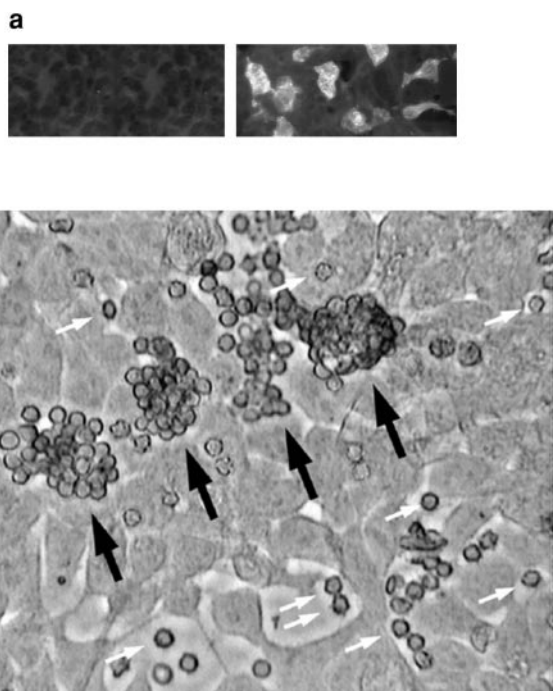

FIG. 3. Hemagglutinin expression. Cells from the 293 cell line were transfected by recombinant DNA and expression was evaluated by immunofluorescence (a) or by hemadsorption (b). Immunofluorescence (a): untransfected control (left) and cells transfected by a plasmid encoding recombinant HA (right). The cells have been incubated with mouse monoclonal antibodies to influenza HA, followed by incubation with FITC-labeled anti-mouse IgG. Cells that express HA appear green under UV illumination. Hemadsorption (b) of guinea pig erythrocytes to HEK293 cells transfected with influenza HA in the pUACMVSMI2 DNA vector. Black arrows indicate clumps of guinea pig erythrocytes adsorbed to HA-expressing HEK293 cells, whereas white arrows show several single erythrocytes distributed over the lawn of untransfected 293 cells. The figure shows a phase contrast image of live cells in culture, where erythrocytes appear naturally red.

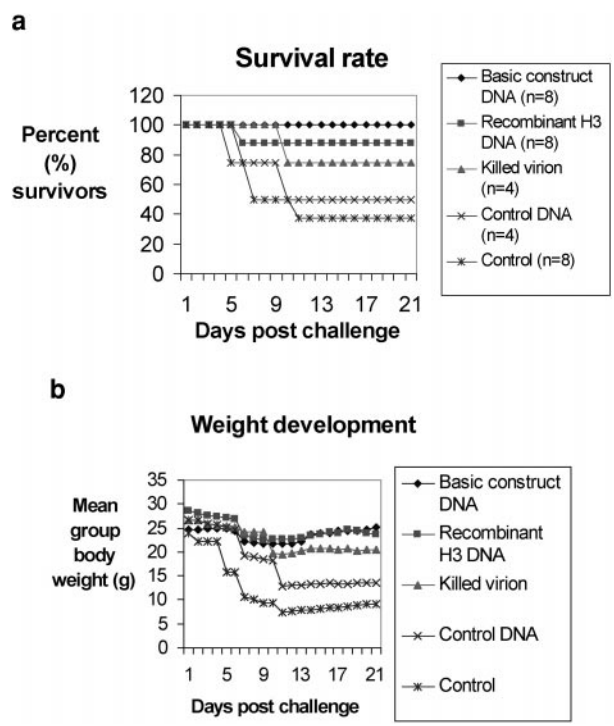

FIG. 4. Challenge data. Vaccinated mice were challenged with the heterologous influenza A/PR/8/34(H1N1) virus. Survival (a) and body weight loss (b) were observed 20 days postchallenge. Mice immunised with DNA have the highest survival rate and the highest mean group body weight after 20 days.

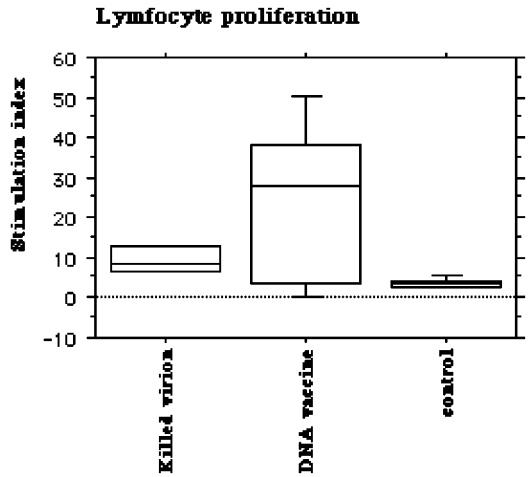

FIG. 5. Box plot illustrating the lymphocyte proliferation stimulation index (SI) 10th, 25th, 50th (median), 75th, and 90th percentiles after stimulation of lymphocytes from mice immunised with a recombinant HA gene encoding the variable region from the A/Stockholm/5/99(H3N2) virus ($n = 5$, responders: 4/5) and mice immunised with control DNA (the HIV-1 rev gene, $n = 5$, responders = 0) with the influenza A/Stockholm/5/99(H3N2) virus. Only the 25th, 50th (median), and 75th percentiles are shown in the mice immunised with killed influenza A/Stockholm/5/99(H3N2) ($n = 4$, responders:4/4). Responders are defined as having a SI>3 and a net cpm>1000.

antigens was observed after immunisation with NP, HA and M_1 encoding plasmids (Donnelly *et al.,*1997). However, protection against the drifted antigen seemed to be independent of the HA-encoding plasmid because immunisation with only the NP- and M_1-encoding plasmids also gave significantly better protection than protein vaccination. There is reason to believe that a DNA vaccine might have a greater ability to elicit a cellular immune response than a killed virion vaccine (Robinson, 1997). The foreign gene is endogenously expressed and the proteins subsequently processed, thus mimicking antigen processing during viral infection.

The ability of DNA vaccines to elicit a cellular immune response is also indicated by our lymphocyte proliferation data where an influenza specific immune response was primed following a single immunisation with a HA-encoding DNA vaccine. The lymphocyte proliferation assay gives information about priming of an immune response, even if antibody production and/or CTL responses may still be low. Indeed, antibodies were not detectable in sera after a single immunisation with the DNA vaccine despite a strong T-cell response in the lymphocyte proliferation assay.

In this study our objective was to create a HA DNA vaccine that could be rapidly modified in accordance with the antigenic drift of the influenza virus. Even though a DNA vaccine may elicit an immune response with an enhanced capability to protect against heterologous viruses by inducing a T helper 1 (Th1) type response, we believe that highly specific neutralising antibodies to HA are important for optimal protection. This is relevant if only the HA gene is considered for immunisation because neutralising antibodies are important for preventing infection, while cytotoxic T lymphocytes may limit the severity of systemic disease. Our challenge results indicate that DNA immunisation gave a higher degree of protection against challenge by a heterologous influenza strain than did protein vaccination even though this remains to be thoroughly investigated, for instance, in a primate model. This may reflect an important role of cellular immunity.

Hemagglutinin-specific B- and T-cell responses were demonstrated, and we have thus obtained an immunogenic vaccine that appears to elicit a unique combination of both specific B cells and broadly reactive T cells. The immunisation and challenge experiments were undertaken to compare the protective efficacy of our DNA vaccine vector versus a homologous killed virus vaccine in a mouse model system. No information regarding cross protection between H3 subtypes could be gathered from the present experiments, but it was clear from the challenge experiments that immunisation with the full-length H3 gene protected against challenge with the mouse-adapted A/PR/8/34 (H1N1) strain. H3 subtype cross-protection, therefore needs to be studied either in a different, susceptible animal model, such as the ferret (Donnelly *et al.,*1997) or, for *in vitro* studies of T-cell reactivity, using constructs encoding isolated variable domains of the H3 gene. Such experiments are in progress.

MATERIALS AND METHODS

RNA- and virus isolation

MDCK cells were used to culture six influenza strains: A/Netherlands/18/94(H3N2), a macaque-adapted strain kindly donated by G. Rimmelzwaan and A. Osterhaus; A/Umeå/1/95(H3N2), which is A/Johannesburg/33/94-like; A/Stockholm/6/96(H3N2), which is A/Wuhan/359/95-like; A/Stockholm/7/97(H3N2), which is A/Sydney/5/97-like; and A/Stockholm/5/99(H3N2), which is A/Sydney/5/97-like; and the A/PR/8/34 (H1N1) strain. Total RNA from infected cells was extracted with standard techniques according to Chomczynski and Sacchi (1987). The virus-containing supernatant (10^7 $TCID_{50}$/ml) from the pelleted influenza-infected MDCK cells was used for virus isolation and vaccine production. PEG 6000 (10% w/v) was added to the supernatant and incubated at 4°C overnight followed by ultracentrifugation at 28,000 rpm at 4°C for 16 h in a sucrose gradient of 30–60%. The gradient was fractionated in 1-ml fractions, and the virion-containing fractions were identified by indirect ELISA with the H3-specific monoclonal antibody (MC3A10–18, WHO Collaborating Center for Virus and Influenza and Research, Lyon, France) to HA and a horseradish peroxidase conjugated anti-mouse IgG antibody (Dako, Copenhagen, Denmark). The virus-containing fractions were pooled

and inactivated by adding an equal volume of 4% paraformaldehyde in PBS for 2 h. The virus-PFA/PBS suspension was absorbed with aluminum phosphate at 4°C overnight before inoculation into the mice.

RT–PCR

The viral RNA precipitate was pelleted and resuspended in water and used for reverse transcription with random 14-mer primers. Enzymes and buffers were from Boehringer Mannheim (Mannheim, Germany).

Two primers were designed using consensus sequences from the HA(H3) gene to yield a PCR product of 1048 bp from the HA region of H3 influenza strains. The sense primer sequence was: 5′-CTATCATTGCTTTGAGCTAC-3′ and the antisense primer sequence was: 5′-CTTCCCAACCATTTTCTATG-3′ (Life Technologies, Täby, Sweden).

PCR amplification of the HA gene from the reverse transcription mixture was performed using an annealing temperature of 52°C and 30 cycles of amplification. Enzymes, buffers, and PCR machines were from Perkin–Elmer (Foster City, Ca).

Cloning through homologous recombination

The basic construct was created by cloning the HA gene from the influenza A/Stockholm/6/96/(H3N2) into the pUA CMV SMI2 vector (Fig. 1) by standard procedures (Sambrook *et al.,* 1989). The variable domain of the HA gene in the basic construct was replaced with the HA gene from the isolates described (Fig. 1). Basic construct DNA (10 μg) was cleaved with *Eco*81I (MBI Fermentas, Vilnius, Lithuania) and *Xba*I (Boehringer Mannheim), thus creating a vector handle. The vector handle was isolated from the 440-bp *Eco*81I-*Xba*I fragment by agarose gel electrophoresis and purified. The linear vector handle (1–5 μg) and the PCR fragment (1–5 μg) were used for heat shock transformation (Alberts *et al.,* 1994; Hanahan *et al.,* 1995) into competent *Escherichia coli* XL1 blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacIqZDM15 Tn10 (Tet^r)], Stratagene, La Jolla, Ca}.

Sequence analysis

Recombinants were analysed by sequencing (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit) on an ABI Prism 310 Genetic Analyzer (Perkin–Elmer). Two vector-specific sequencing primers were designed, the promoter-specific sense primer: 5′-GAGGGGCGGCGACTGAATG-3′ and the polyA site specific anti sense primer: 5′-TTAGTAGGTGTTGAAACAATAAGT-3′.

Expression of recombinant hemagglutinin

Cells from the 293 cell line (human embryonic kidney cells transformed with adenovirus 5 E1A and E1B genes) (Graham and van der Eb, 1973) were transfected using calcium phosphate-DNA precipitate according to Chen and Okayama (1987). The cells were cultured in IMDM (Life Technologies). Medium was changed 24 h after transfection, and the cells were harvested after 48 h.

Transfected cells were assayed for gene expression by immunofluorescence staining of HA with an H3-specific mAb to HA and a secondary FITC-conjugated anti-mouse IgG antibody (Chemicon AB, Malmö, Sweden).

The ability of the transfected cells to cause hemadsorption was assayed by addition of red blood cells (0.4%) from guinea pigs. Hemadsorption could be detected by light microscopy as adherence of erythrocytes to the surface of transfected cells.

Immune responses and challenge

The plasmid DNA vaccines were produced in *E. coli* XL1 blue and purified by QIAGEN Megaprep (QIAGEN, Hilden, Germany). Fifty micrograms of DNA was given as a primary immunisation (25 μg in each hind leg) to C57BL/6 mice. Approximately 10 μg of inactivated virus protein (5 μg in each hind leg) was used to immunise C57BL/6 mice by intramuscular (im) injection. The antibody titres (specific IgG and IgM) of the immunised mice were measured in wells coated with the appropriate antigen [A/Stockholm/7/97(H3N2) or A/Stockholm/5/99 (H3N2)] in an indirect ELISA. Briefly, the microtiter wells were coated with 100 μl antigen dissolved in PBS in a 1/300 dilution overnight at room temperature. The wells were washed with 0.9% NaCl solution containing 0.05% Tween 20, and 100 μl serum diluted 1/50 or 1/500 in ELISA buffer (1.25g BSA, 0.12 ml Tween, and 250ml PBS) was added. The plates were incubated at 37°C for 2 h and washed again. HA-specific antibodies were detected with horseradish peroxidase conjugated goat-anti-mouse IgG and IgM antibodies (Dako). After 2 h at 37°C, the plates were washed, and 100 μl OPD substrate was added. The colour reaction was stopped by addition of 2.5M H_2SO_4. The optical density was determined at 540 nm, and antibody titres were defined as the reciprocal of the highest dilution giving a net OD-value higher than two standard deviations above the OD reading of a negative sample.

For the challenge study, eight mice were given the basic construct DNA and eight mice received a plasmid with a recombinant HA gene encoding the variable region from the influenza A/Stockholm/7/97(H3N2). Four mice were given the killed virion vaccine, and four mice were given a control plasmid encoding the HIV-1 rev gene under the CMV immediate early promoter (same dose as for HA encoding DNA vaccine). Another eight mice were not immunised and served as negative controls. Six weeks after primary immunisation, an intramuscular booster dose was administered (same dose as above). Another 6–8 weeks later the mice were inocu-

lated by intranasal instillation (25 μl/nostril) according to Robinson *et al.* (1997) (with the difference being that the mice were not anaesthetised) of saline containing a 100-fold dilution of egg-cultured mouse-adapted influenza A/PR/8/34 virus stock (kindly provided by H. L. Robinson and R. G. Webster). This dose was titrated to be the LD_{50} in inoculated C57BL/6 mice.

Lymphocyte proliferation

On Day 23 postimmunisation, spleen cells were taken from a total of 14 mice immunised once with inactivated influenza A/Stockholm/5/99(H3N2) ($n = 4$, one mouse was killed by its siblings), DNA vaccine encoding the variable region of the same virus ($n = 5$) or with the DNA vaccine vector backbone, which served as an unimmunised control ($n = 5$). The spleens were fragmented and lymphocytes isolated by sedimentation. The lymphocytes were washed once in phosphate buffered saline (PBS, Unimedic, Matfors, Sweden) and then dissolved in RPMI 1640 (Life Technologies) with 10% inactivated foetal calf serum (FCS, Life Technologies), 50 U/ml penicillin, and 50 μg/ml streptomycin and counted in a CX320 cell coulter. The cells were washed and diluted to 2.0×10^6 cells/ml. Cells were aliquoted in a 96-well plate (2.0×10^5/well) with RPMI 1640 and 10% FCS and with 5 μg/ml Con A (Boehringer Mannheim, Mannheim, Germany) as a positive control or influenza A/Stockholm/5/99(H3N2) in dilutions 1:20 and 1:40. The plates were incubated at 37°C for 5 days. After 5 days, 50 μl tritium-labeled thymidine (1μCi) was added to each well, and the plates were incubated at 37°C overnight. The cells were harvested onto nitro-cellulose filters (Wallac, Stockholm, Sweden) and dried overnight at 58°C. The filters were then sealed inside a plastic pocket and wet with 4.5 ml Scint fluid. The level of stimulation was measured as the amount of incorporated [^{3}H]thymidine. A net count per minute (net cpm; the cpm of the cells stimulated by the antigen minus the cpm of cells with medium) exceeding 1000 and a stimulation index (the cpm of the cells stimulated by the antigen divided by cpm of non stimulated cells) >3 were considered to be a specific stimulation response.

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